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Plant carotenoids: genomics meets multi-gene engineering

Giovanni Giuliano

Carotenoids are present in plant photosynthetic tissues, where they have essential roles in photoreception and photoprotection, as well as in non-photosynthetic tissues, where they act as colorants, precursors for plant isoprenoid volatiles and signaling molecules (abscisic acid and strigolactones), nutritional antioxidants and vitamin A precursors. This review presents the recent advances in our understanding of their biosynthesis, the key metabolic steps controlling their accumulation in plant non-photosynthetic tissues and their metabolic engineering using multi-gene approaches.

Addresses
Italian National Agency for New Technologies, Energy and Sustainable Development (ENEA), Casaccia Research Center, Via Anguillarese 301, Roma 00123, Italy

Corresponding author: Giuliano, Giovanni (giovanni.giuliano@enea.it)

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Introduction

Carotenoids are C40 isoprenoid compounds, made of C5 isopentenyl building blocks, which in plants are synthesized through two separate pathways: the cytoplasmic mevalonate pathway and the plastid-located (or MEP) pathway (Figure 1a). The MEP pathway provides a large part of the carbon flux that goes into carotenoids [1]. Its main products, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), are condensed into geranylgeranyl pyrophosphate (GGPP), which acts as a precursor for a series of isoprenoid molecules, including carotenoids (Figure 1a). The condensation of two GGPP molecules yields 15-*cis*-phytoene, a colorless, linear carotene, which is converted into lycopene by a poly-*cis* desaturation/isomerization pathway relying on two desaturases and two isomerases. Lycopene is the substrate of two competing cyclases, introducing β-rings and ε-rings at the ends of the molecule. A series of heme and non-heme hydroxylases, one epoxidase and one de-epoxidase, complete the canonical plant pathway. Some steps (the biosynthesis of neoxanthin, the 9-*cis* isomerization of neoxanthin and violaxanthin) await complete elucidation. An excellent review on the mechanistic aspects of carotenoid biosynthesis was published recently [2].

Carotenoids are the substrates of cleavage enzymes, which come in at least two types (Figure 1): nine-*cis*-epoxycarotenoid dioxygenases (NCEDs) that catalyze the first step in abscisic acid (ABA) biosynthesis, and carotenoid cleavage dioxygenases (CCDs) that catalyze a vast array of different cleavage steps giving rise to both volatile and non-volatile apocarotenoids, with functions as aromas, allelopathic substances or hormone-like substances (strigolactones) [3].

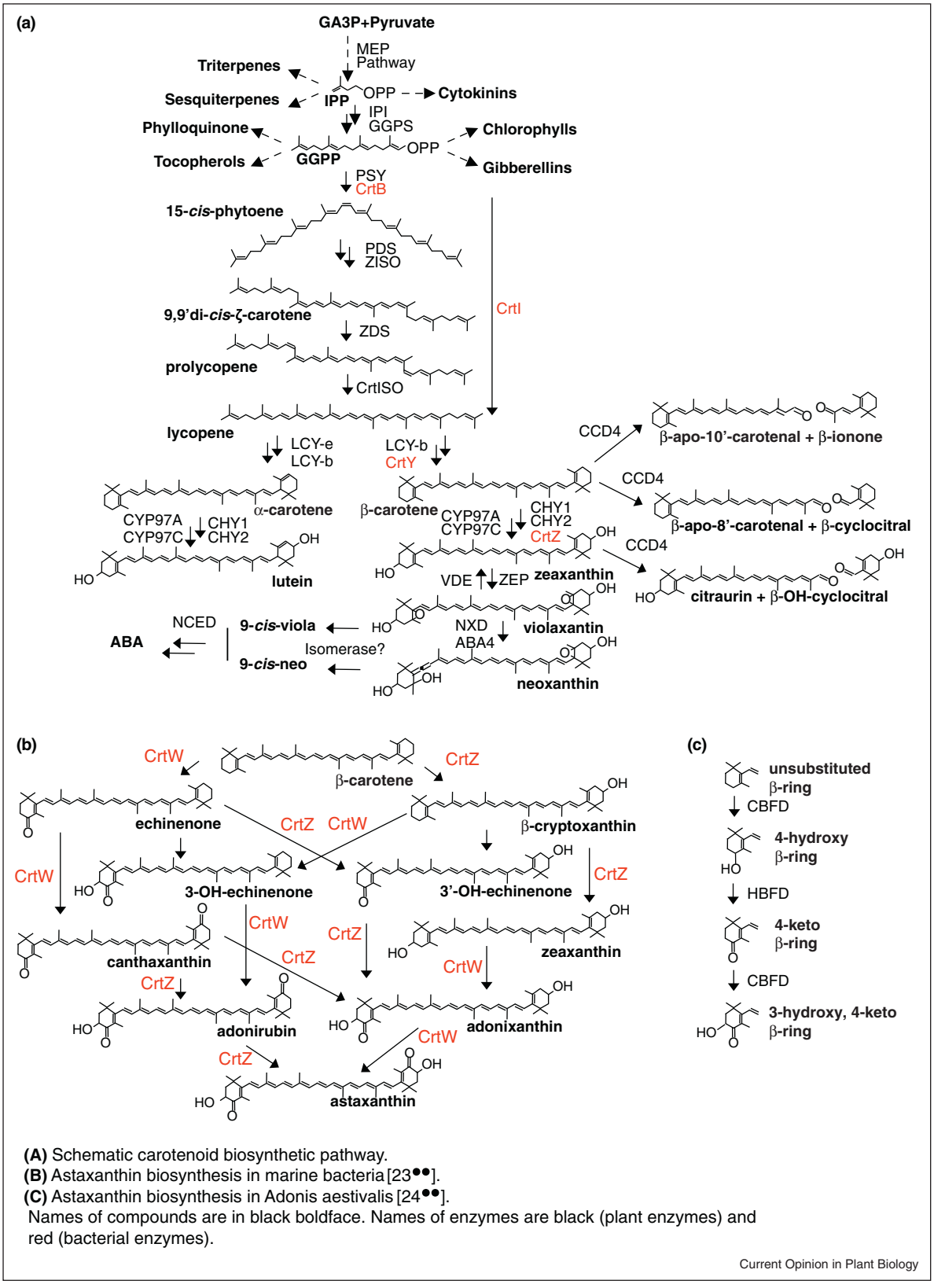
Metabolic engineering of plant carotenoid biosynthesis has focused primarily on two targets. One target is β-carotene, a nutritional vitamin A precursor, for alleviating vitamin A deficiency in populations whose diet is deficient in this vitamin. These biofortification efforts, utilizing both transgenic and classical breeding approaches, have focused on staple foods that are deficient in β-carotene, such as rice, white maize, potato and cassava [4••,5•,6,7••,8•,9•,10•].

Additional targets are high value xanthophylls used as food and feed additives. Certainly the most explored one is astaxanthin, a dihydroxy, diketo xanthophyll (Figure 1) synthesized by many marine bacteria and algae and by a handful of plants and fungi, used both as a dietary antioxidant and as a feed additive in fisheries. Astaxanthin is presently produced through chemical synthesis or extraction from microalgae, and alternative sources are needed to meet market demand at competitive prices. Transgenic and transplastomic approaches for the production of astaxanthin *in planta* have focused on tobacco, carrot, tomato, potato and maize [7••,11•,12,13,14,15•,16•17,18].

Updating the carotenoid pathway

In organisms with oxygenic photosynthesis (cyanobacteria and plants) the conversion of 15-*cis*-phytoene to lycopene is effected through a poly-*cis* pathway, involving the action of two desaturases introducing *cis* double-bonds and two isomerases converting them back to the all-*trans* configuration (Figure 1a). Phytoene desaturase (PDS), ζ-carotene desaturase (ZDS) and prolycopene isomerase (CrtISO) are flavoproteins catalyzing non-redox reactions [19,20], like the bacterial CrtI enzyme, which is however able to perform all the reactions

Figure 1



(A) Schematic carotenoid biosynthetic pathway.
(B) Astaxanthin biosynthesis in marine bacteria [23••].
(C) Astaxanthin biosynthesis in Adonis aestivalis [24••].
Names of compounds are in black boldface. Names of enzymes are black (plant enzymes) and red (bacterial enzymes).

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required for lycopene synthesis [21•]. This catalytic versatility of *CrtI* has prompted its use in many metabolic engineering efforts. A second plant isomerase, ζ-carotene isomerase or ZISO, able to isomerize the 15-*cis* double bond, was recently isolated [22••]. *ZISO* encodes a small protein, very different from CrtISO, and contains consensus sequences for iron binding.

The biosynthesis of astaxanthin from β-carotene in marine bacteria was elucidated over 15 years ago [23••]. It is mediated by two genes, *CrtZ* and *CrtW*, encoding a hydroxylase and a ketolase, respectively, acting in a combinatorial fashion on the two β-rings (Figure 1b). The pathway for astaxanthin biosynthesis in flowers of the higher plant *Adonis aestivalis* was recently elucidated [24••] (Figure 1c): first, a β-ring 4-dehydrogenase (CBFD) catalyzes the formation of a 4-hydroxyl group, which is then converted to a 4-keto group by a hydroxy-β-ring 4-dehydrogenase (HBFD); finally, CBFD inserts a 3-hydroxyl group, completing the biosynthesis. This pathway has been shown to be active in *Escherichia coli*; in contrast to the bacterial enzymes, which seem to act in a competitive fashion when modifying the same ring, leading to the accumulation of intermediates [25], the plant enzymes seem to act synergistically, leading to complete conversion of β-carotene into astaxanthin. Despite this, to date there are no examples of transfer of the *Adonis* pathway to other flowering plants.

Genomics comes of age

Potato and tomato have been extensively utilized in the past for metabolic engineering efforts. Sequencing of their genomes [26•,27••] unveiled several novel genes in carotenoid biosynthesis and their regulation. In both plants, the first dedicated enzyme of the pathway, phytoene synthase (PSY) (Figure 1a), is encoded by three genes, two of which (*PSY1* and *PSY2*) show expression in most tissues, while expression of *PSY3* is very low and confined to roots. The *PSY1–PSY2* pair was generated by the *Solanum* whole genome triplication dating back to approximately 70 million years ago (mya), while the origin of *PSY3* seems more ancient [27••]. The expression pattern of the *Solanum PSY1* remained generally similar to that of its *PSY2* counterpart until recently (0.5–1 mya), when its overexpression in fruits gave rise to the lycopene accumulation pattern characteristic of the red fruits of tomato and *S. pimpinellifolium* (G Falcone and G Giuliano, unpublished data). *PSY* and *ZISO* are the two most highly expressed genes in the carotenoid pathway in fruits of red-fruited species, while they are expressed at low levels in fruits of green-fruited species (G Falcone and G Giuliano, unpublished data). *PSY1* is absolutely necessary for lycopene accumulation in tomato fruits, while the function of *ZISO* can be partially substituted by light [28•].

PSY is a key regulator of carotenoid accumulation in staple crops such as maize [29•], durum wheat [30] and cassava [10•]. A single nucleotide polymorphism in a *PSY* gene highly expressed in cassava roots co-segregates with high β-carotene levels. This polymorphism results in a single amino acid change in a highly conserved region of the protein which, when tested in *E. coli*, results in increased catalytic activity. A systematic comparison showed that *PSY* genes from different plant sources differed greatly in their capacity to induce accumulation of β-carotene in the endosperm of golden rice, with rice and maize PSY being the most efficient [5•]. A recent survey of several PSY enzymes from different sources showed that the majority of them localize to plastoglobules, with the exception of maize PSY1, which localizes preferentially in the stroma [31•].

PSY seems to be a key integrator for several signals regulating the carotenoid pathway. In *Arabidopsis*, the phytochrome-interacting transcription factor PIF1 directly interacts with the promoter of the *PSY* gene, mediating its light-regulated transcription [32•]. In tomato, the MADS box regulator of ripening, RIN, interacts with the *PSY* promoter [33,34,35]. Overexpression in potato tubers of the cauliflower Or regulator, a molecular chaperone able to induce formation of chromoplasts in heterologous systems [36], results in increased PSY stability [37]. The tomato Stay Green SlSGR1 protein regulates fruit lycopene and β-carotene accumulation through direct interaction with the PSY enzyme [38]. Finally, it has been proposed that epistasis in tomato color mutations involves regulation of *PSY1* expression by *cis*-carotenoids [39•].

β-carotene hydroxylase (CHY) and lycopene ε-cyclase (LCY-e) are important regulators of β-carotene and total carotenoid accumulation in maize. Mutations at the *LCY-e* locus redirect the flux towards the β-branch, increasing β-carotene content [40•]; *CHY* alleles associated with reduced transcript expression correlate with higher β-carotene concentrations [9•,41]. In potato, transgenic silencing of the *CHY* and *LCY-e* genes results in increased β-carotene and total carotenoid content [42,43]. A dominant allele of *CHY2* is associated with accumulation of carotenoids, especially β-xanthophylls, in potato [44•] and the *CHY2* gene is more expressed in the high-carotenoid DM potato genotype than in the low carotenoid RH one [26•]. A second gene mainly controlling the accumulation of zeaxanthin in potato is zeaxanthin epoxidase (*ZEP*) (Figure 1). Loss-of-function alleles of *ZEP*,

(Fig.1 legend continue) **(a)** Schematic carotenoid biosynthetic pathway. **(b)** Astaxanthin biosynthesis in marine bacteria [23••]. **(c)** Astaxanthin biosynthesis in Adonis aestivalis [24••]. Names of compounds are in black boldface. Names of enzymes are black (plant enzymes) and red (bacterial enzymes).

or silencing of the *ZEP* gene, cause an accumulation of zeaxanthin [44•,45].

Sequencing of the peach genome [46•] helped clarify the molecular basis of the high-carotenoid, yellow flesh trait in peach. White flesh is dominant over yellow in peach, and is associated with increased emission of isoprenoid volatiles, likely derived from the cleavage of carotenoid molecules, suggesting that the trait is due to a gain-of-function mutation in a carotenoid cleavage dioxygenase. Analysis of a yellow-fleshed cultivar and of its white-fleshed mutant showed that a *CCD4* transcript was induced in the mutant [47•]. Analysis of a larger genotype collection suggested that the white flesh color (active *CCD4*) was the ancestral situation in peach and that yellow-fleshed genotypes arose in modern cultivars through three independent mutational mechanisms (nucleotide substitutions, small insertions and transposable element insertions) [48•].

CCD4 is also a key determinant of carotenoid pigmentation in petals of *Chrysanthemum* [49] and of *Lilium* [50]. In contrast, in *Calendula* petals *CrtISO* is a key determinant [51•]. *CCD4* is also a key determinant of carotenoid/apocarotenoid content in potato tubers [52], *Arabidopsis* seeds and senescing leaves [53•] and *Citrus* fruits [54•]. In *Arabidopsis* and potato, *CCD4* levels correlate inversely with pigmentation, while in *Citrus* the opposite is true. This is because *Citrus* CCD4 asymmetrically cleaves β-carotene and zeaxanthin at the 7,8 position, giving rise to non-volatile C30 apocarotenoids (Figure 1a), which accumulate in the flavedo of fruits and contribute to their pigmentation. In potato, RNAi inhibition of *CCD4* caused not only accumulation of carotenoids, but also a series of developmental phenotypes [52], suggesting the intriguing hypothesis that a CCD4 cleavage product may have a signaling role, or that *CCD4* silencing in tubers re-routes the flux towards other cleavage pathways with a signaling role (NCED, giving rise to ABA and/or CCD7/8, giving rise to strigolactones).

Multiple gene engineering

The stable overexpression of multiple transgenes is one of the main bottlenecks limiting reconstruction of complex biochemical pathways. Early examples of mini-pathway engineering in rice, tomato and potato employed 2–3 transgenes cloned in a single *Agrobacterium* construct [4••,5•,6,55]. More recently, up to seven different bacterial carotenoid genes (from isopentenyl pyrophosphate isomerase (*IPI*) to *CrtW*, Figure 1), have been assembled in a single *Agrobacterium* construct and used to transform canola and *Lilium* [56,57].

A second approach for mini-pathway engineering is combinatorial transformation of independent gene constructs. Five independent transgenes, from *PSY* to *CrtW* (Figure 1), were introduced in a white endosperm maize using biolistic transformation [7••]. Different combinations of expressed transgenes, from none to all five, were recovered in an approximately normal distribution. This approach was brought one step further by stacking two carotenoid genes and one gene each from the ascorbate and folate pathways to obtain maize simultaneously fortified with β-carotene (provitamin A), vitamin C and folate [8•].

A third approach is the expression of different genes (*CrtB* and *CrtI*) as a single transcript under the control of a single promoter [58•]. The downstream gene product is expressed either by inserting an internal ribosomal entry site (IRES) from a plant virus, or by inserting between the two proteins a self-cleaving protein sequence (2A) from an animal virus. The polyprotein construct is ninefold more efficient than the IRES construct in rice endosperm [58•] and has been used to increase total carotenoid and β-carotene levels in soybean seeds [59].

A fourth approach, applicable in principle to any plastid-localized biosynthetic pathway, is the expression of multiple genes as a single operon inserted in plastid DNA. This approach can be used to insert biosynthetic pathways from a different organism, or from a different cell compartment, to plastids: the *CrtZ* and *CrtW* genes for astaxanthin biosynthesis have been overexpressed in tobacco [15•] and lettuce [17]. In a separate approach, the whole cytoplasmic mevalonate pathway for biosynthesis of IPP was transferred to tobacco plastids [60•]. The functionality of the transferred pathway was demonstrated through resistance to fosmidomycin (an inhibitor of the MEP pathway). Transplastomic plants accumulated higher levels of carotenoids, sterols and triacylglycerols than control plants.

In general, the carotenoids accumulated in plants engineered with multiple genes are consistent with the function of the introduced transgenes, with a series of interesting exceptions:

(i) The multifunctional CrtI desaturase/isomerase utilized in many metabolic engineering experiments is unable to catalyze complete conversion of phytoene into downstream compounds [6,7••], suggesting that part of the phytoene pool is inaccessible to CrtI. Fractionation of transgenic tomato chromoplasts overexpressing *CrtB* + *CrtI* showed that phytoene was mainly found in plastoglobules, whereas the CrtB and CrtI enzymes were localized to the membrane fraction [61•]. A series of enzymes for carotenoid metabolism are known to localize to plastoglobules, including ZDS, LCY-b, CHY and CCD4 [62,63], whereas PSY1 has been reported to be localized in both plastoglobules and stroma [31•,61•].

(ii) A lycopene β-cyclase (*LCY-b*) transgene is not required for β-carotene accumulation in different plant tissues (tomato fruits and maize and rice endosperm). All three tissues overexpressing *CrtB*/*PSY* + *CrtI* accumulate significant levels of β-carotene, [4••,7••,61•]. Endogenous *LCY-b* expression is probably the cause of this phenotype.

(iii) In plants transformed with multiple bacterial genes for the production of astaxanthin, the biosynthesis is often arrested at intermediate compounds, such as echinenone, 3′-OH-echinenone, canthaxanthin and adonixanthin [7••,56,57] (Figure 1b). This limitation can be overcome by the use of algal ketolases and hydroxylases showing co-operative, rather than antagonistic action [18,64,65] or by plastid transformation [15•,17].

Concluding remarks

In recent years, the availability of genomic tools, including whole genome sequences, has uncovered a series of quantitative trait loci (QTLs) controlling carotenoid accumulation in agricultural plants. These QTLs can now be used in breeding programs aimed at optimizing plant carotenoid composition. At the same time, advanced tools for engineering of the nuclear and plastid genomes have been developed, including combinatorial nuclear transformation [7••] and artificial *cis*-elements for the expression in chromoplasts [66•]. The additional availability of synthetic biology tools [67] and metabolic models accounting for subcellular compartmentalization and tissue-specificity [68] will dramatically improve our capacity to manipulate plant carotenoid biosynthesis for biofortification and green chemistry applications.

Note added in proof

A gene essential for neoxanthin biosynthesis was recently cloned from tomato [69].

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